FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
1038-1191MIS:jb	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	
09/914621	
INTERNATIONAL APPLICATION NO. PCT/CA00/00227	INTERNATIONAL FILING DATE March 3, 2000
PRIORITY DATE CLAIMED March 5, 1999	

TITLE OF INVENTION
NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

APPLICANT(S) FOR DO/EO/US
Xiaomao Li; et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). - unsigned copy
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Initial Information Data Sheet

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/914621	PCT/CA00/00227	1038-1191MIS:jb

24. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$710.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	24 - 20 =	4	x $18.00	$72.00
Independent claims	4 - 3 =	1	x $80.00	$80.00
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00
TOTAL OF ABOVE CALCULATIONS =				$1,012.00
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$0.00
SUBTOTAL =				$1,012.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	$0.00
TOTAL NATIONAL FEE =				$1,012.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	$0.00
TOTAL FEES ENCLOSED =				$1,012.00
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$1,012.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **192253** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Michael I. Stewart
Sim & McBurney
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Toronto, Ontario
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24223
PATENT TRADEMARK OFFICE

SIGNATURE

Michael I. Stewart
NAME

24,973
REGISTRATION NUMBER

August 30, 2001
DATE

Sim & McBurney

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December 21, 2001

VIA COURIER

The Commissioner of Patents
and Trademarks
Washington, D.C. 20231
U.S.A.

Dear Sirs:

RE: United States Patent Application No. 09/914,621
Applicant: Xiaomao Li et al.
Title: NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

With reference to your letter mailed October 15, 2001, submitted herewith are:

1. Declaration and Power of Attorney document duly executed by the applicants.

2. Cheque in the prescribed fee for surcharge fee and petition fee (see below).

Petition is hereby made under the provisions of 37 C.F.R. 1.136(a) for an extension of one month for the period for submission of the Declaration and Power of Attorney.

Yours truly,

Michael I. Stewart
Registration No. 24,973

Enclosure(s)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re National Phase of International

Appl'n. No.	:	PCT/CA00/00227
Filed	:	March 3, 2000
Applicant	:	Xiaomao Li, et al.
Title	:	NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES
Docket No.	:	1038-1191 MIS:jb

August 30, 2001

BY COURIER

The Commissioner of Patents
and Trademarks,
Washington, D.C. 20231,
U.S.A.

PRELIMINARY MENDMENT

Sir:

Please amend the above-identified application as follows:

In the Specification:

Before the first line of the specification, add the following:

" REFERENCE TO RELATED APPLICATIONS

This application is a national phase application under 35 U.S.C. 371 of PCT/CA00/00227."

REMARKS/ARGUMENTS

The specification has been amended on page 1 to reflect that this application is a U.S. National Phase filing under 35 U.S.C. 371 of PCT/CA00/00227.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**Version with markings to show changes made**."

Respectfully submitted,
SIM & McBURNEY

M.I. Stewart
Reg. No. 24,973

Toronto, Ontario, Canada,
(416) 595-1155
FAX No. (416) 595-1163

Appl. No.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Before the first line of the specification, add the following:

" REFERENCE TO RELATED APPLICATIONS

This application is a national phase application under 35 U.S.C. 371 of PCT/CA00/00227."

TITLE OF INVENTION

NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

FIELD OF INVENTION

The present invention is related to the field of Respiratory Syncytial Virus (RSV) vaccines and is particularly concerned with vaccines comprising nucleic acid sequences encoding the fusion (F) protein of RSV.

BACKGROUND OF INVENTION

Respiratory syncytial virus (RSV), a negative-strand RNA virus belonging to the *Paramyxoviridae* family of viruses, is the major viral pathogen responsible for bronchiolitis and pneumonia in infants and young children (ref. 1 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Acute respiratory tract infections caused by RSV result in approximately 90,000 hospitalizations and 4,500 deaths per year in the United States (ref. 2). Medical care costs due to RSV infection are greater than $340 M annually in the United States alone (ref. 3). There is currently no licensed vaccine against RSV. The main approaches for developing an RSV vaccine have included inactivated virus, live-attenuated viruses and subunit vaccines.

The F protein of RSV is considered to be one of the most important protective antigens of the virus. There is a significant similarity (89% identity) in the amino acid sequences of the F proteins from RSV subgroups A and B (ref. 3) and anti-F antibodies can cross-neutralize viruses of both subgroups as well as protect immunized animals against infection with viruses from both subgroups (ref. 4). Furthermore, the F protein has been identified as a major target for RSV-specific cytotoxic T-lymphocytes in mice and humans (ref. 3 and ref. 5).

The use of RSV proteins as vaccines may have obstacles. Parenterally administered vaccine candidates have so far proven to be poorly immunogenic with regard to the induction of neutralizing antibodies in seronegative humans or chimpanzees. The serum antibody response induced by these antigens may be

further diminished in the presence of passively acquired antibodies, such as the transplacentally acquired maternal antibodies which most young infants possess. A subunit vaccine candidate for RSV consisting of purified fusion glycoprotein from RSV infected cell cultures and purified by immunoaffinity or ion-exchange chromatography has been described (ref. 6). Parenteral immunization of seronegative or seropositive chimpanzees with this preparation was performed and three doses of 50 μg were required in seronegative animals to induce an RSV serum neutralizing titre of approximately 1:50. Upon subsequent challenge of these animals with wild-type RSV, no effect of immunization on virus shedding or clinical disease could be detected in the upper respiratory tract. The effect of immunization with this vaccine on virus shedding in the lower respiratory tract was not investigated, although this is the site where the serum antibody induced by parenteral immunization may be expected to have its greatest effect. Safety and immunogenicity studies have been performed in a small number of seropositive individuals. The vaccine was found to be safe in seropositive children and in three seronegative children (all > 2.4 years of age). The effects of immunization on lower respiratory tract disease could not be determined because of the small number of children immunized. One immunizing dose in seropositive children induced a 4-fold increase in virus neutralizing antibody titres in 40 to 60% of the vaccinees. Thus, insufficient information is available from these small studies to evaluate the efficacy of this vaccine against RSV-induced disease. A further problem facing subunit RSV vaccines is the possibility that inoculation of seronegative subjects with immunogenic preparations might result in disease enhancement (sometimes referred to as immunopotentiation), similar to that seen in formalin inactivated RSV vaccines. In some studies, the immune response to immunization with RSV F protein or a synthetic RSV FG fusion protein resulted in a disease enhancement in rodents resembling that induced by a formalin-inactivated RSV vaccine. The association of immunization with disease enhancement using non-replicating antigens suggests caution in their use as vaccines in seronegative humans.

Live attenuated vaccines against disease caused by RSV may be promising for two main reasons. Firstly, infection by a live vaccine virus induces a balanced immune response comprising mucosal and serum antibodies and cytotoxic T-

lymphocytes. Secondly, infection of infants with live attenuated vaccine candidates or naturally acquired wild-type virus is not associated with enhanced disease upon subsequent natural reinfection. It will be challenging to produce live attenuated vaccines that are immunogenic for younger infants who possess maternal virus-neutralizing antibodies and yet are attenuated for seronegative infants greater than or equal to 6 months of age. Attenuated live virus vaccines also have the risks of residual virulence and genetic instability.

Injection of plasmid DNA containing sequences encoding a foreign protein has been shown to result in expression of the foreign protein and the induction of antibody and cytotoxic T-lymphocyte responses to the antigen in a number of studies (see, for example, refs. 7, 8, 9). The use of plasmid DNA inoculation to express viral proteins for the purpose of immunization may offer several advantages over the strategies summarized above. Firstly, DNA encoding a viral antigen can be introduced in the presence of antibody to the virus itself, without loss of potency due to neutralization of virus by the antibodies. Secondly, the antigen expressed *in vivo* should exhibit a native conformation and, therefore, should induce an antibody response similar to that induced by the antigen present in the wild-type virus infection. In contrast, some processes used in purification of proteins can induce conformational changes which may result in the loss of immunogenicity of protective epitopes and possibly immunopotentiation. Thirdly, the expression of proteins from injected plasmid DNAs can be detected *in vivo* for a considerably longer period of time than that in virus-infected cells, and this has the theoretical advantage of prolonged cytotoxic T-cell induction and enhanced antibody responses. Fourthly, *in vivo* expression of antigen may provide protection without the need for an extrinsic adjuvant.

In WO 96/04095 published December 19, 1996 and US Patents Nos. 5,843,913, 5,880,104, 6,019,980 and 6,022,864, assigned to the assignee hereof and the disclosures of which is incorporated herein by reference, there is described the provision of non-replicating vectors, specifically plasmid vectors, and immunogenic compositions comprising the same, for administration to a host to generate an immune response to RSV. Such vectors comprise a first nucleotide sequence encoding an RSV F protein or RSV F protein fragment that generates antibodies

and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein; a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host, and a second nucleotide sequence located between the first nucleotide sequence and promoter sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host.

The ability to immunize against disease caused by RSV by administration of a DNA molecule encoding an RSV F protein was unknown before the invention described in the above-mentioned WO 96/04095. In particular, the efficacy of immunization against RSV induced disease using a gene encoding a secreted form of the RSV F protein was unknown. Infection with RSV leads to serious disease. It would be useful and desirable to provide isolated genes encoding RSV F protein and improved vectors for *in vivo* administration for use in immunogenic preparations, including vaccines, for protection against disease caused by RSV and for the generation of diagnostic reagents and kits. In particular, it would be desirable to provide improved vaccines that are immunogenic and protective in humans, including seronegative infants, that do not cause disease enhancement (immunopotentiation).

SUMMARY OF INVENTION

The present invention relates to a method of immunizing a host against disease caused by respiratory syncytial virus, to nucleic acid molecules used therein, and to diagnostic procedures utilizing the nucleic acid molecules. In particular, the present invention is directed towards the provision of improved nucleic acid respiratory syncytial virus vaccines.

In accordance with one aspect of the invention, there is provided an immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a vector which is non-replicating in the host to which it is administered, including a plasmid vector, comprising:

a nucleotide sequence encoding an RSV F protein lacking an autologous RSV F signal peptide sequence and including, in its place, a sequence encoding a

heterologous signal peptide sequence which enhances the level of expression of the RSV F protein; and

a promoter sequence operatively coupled to the nucleotide sequence for expression of the RSV F protein.

The nucleotide sequence encoding the RSV F protein may be that which encodes an RSV F protein from which the transmembrane region is absent. The lack of expression of the transmembrane region results in a secreted form of the RSV F protein. The nucleotide sequence encoding the RSV F protein may include a portion encoding the mature RSV F protein.

One heterologous signal peptide which has been found useful in providing enhanced *in vivo* expression levels of RSV F protein is the signal peptide of Herpes Simplex Virus I (HSV I) gD. Such enhanced expression levels also lead to improve immunogenicity of the vector at the same dosage level as vectors having the autologous signal peptide sequence, as described in WO 96/04095 referred to above. A vector encoding the RSV F protein and provided by this aspect of the invention may specifically be plasmid p82M35B, as seen in Figure 10.

The vector may contain a second nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host. The second nucleotide sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes the RSV F protein. Such second nucleotide sequence may be located between the first nucleotide sequence and the promoter sequence. Such second nucleotide sequence may be that of rabbit β-globin intron II, as shown in Figure 8 (SEQ ID No: 5).

The promoter sequence employed in the vector may be any suitable promoter which provides expression of the RSV F protein in the host. The promoter sequence may be an immediate early cytomegalovirus (CMV) promoter.

Certain of the vectors provided herein may be used to immunize a host against RSV infection or disease by *in vivo* expression of RSV F protein following administration of the vectors in the form of immunogenic compositions.

In accordance with another aspect of the present invention, there is provided an immunogenic composition for *in vivo* administration to a host for the generation

in the host of a protective immune response to RSV F protein, comprising a non-replicating vector as provided herein and a pharmaceutically-acceptable carrier therefor.

In accordance with a further aspect of the present invention, there is provided a method of immunizing a host against disease caused by infection with respiratory syncytial virus, which comprises administering to the host an effective amount an immunogenic composition provided herein.

The invention further extends to the vectors provided herein when used as an immunogen for immunizing a host against disease caused by infection with RSV. In addition, the invention extends to the use of the vectors provided herein in the manufacture of a medicament for immunizing a host against disease caused by RSV.

The present invention also includes a novel method of using a nucleotide sequence encoding an RSV F protein lacking an autologous RSV F signal peptide sequence and including a heterologous signal peptide which enhances the level of expression of RSV F protein, which comprises:

isolating a gene encoding RSV F protein having an autologous RSV F signal peptide sequence;

substituting a nucleotide sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein for the nucleotide sequence encoding the autologous RSV F signal peptide sequence to form the nucleotide sequence,

operatively linking the nucleotide sequence to at least one control sequence to produce a non-replicating vector, said control sequence directing expression of the RSV F protein when said vector is introduced into a host to produce an immune response to the RSV F protein, and

introducing the vector into the host.

The procedure provided in accordance with this aspect of the invention may further include the step of:

operatively linking the nucleotide sequence to an immunoprotection enhancing sequence to produce an enhanced immunoprotection by the RSV F protein in the host, preferably by introducing the immunoprotection enhancing sequence between the control sequence and the nucleotide sequence.

In addition, the present invention includes a method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating a first nucleotide sequence encoding an RSV F protein having an autologous RSV F signal peptide sequence;

substituting a nucleotide sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein for the nucleotide sequence encoding the autologous RSV F signal peptide sequence to form a second nucleotide sequence,

operatively linking the second nucleotide sequence to at least one control sequence to produce a non-replicating vector, including a plasmid vector, the control sequence directing expression of the RSV F protein when introduced into a host to produce an immune response to the RSV F protein when expressed *in vivo* from the vector in a host, and

formulating the vector as a vaccine for *in vivo* administration.

The second nucleotide sequence further may be operatively linked to a third nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host. The vector may be the plasmid vector p82M35B. The invention further includes a vaccine for administration to a host, including a human host, produced by this method as well as immunogenic compositions comprising an immunoeffective amount of the vectors described herein.

As noted previously, the vectors provided herein are useful in diagnostic applications. In a further aspect of the invention, therefore, there is provided a method of determining the presence of an RSV F protein in a sample, comprising the steps of:

(a) immunizing a host with a non-replicating vector as provided herein to produce antibodies specific for the RSV F protein;

(b) isolating the RSV F protein specific antibodies;

(c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and the RSV F protein- specific antibodies; and

(d) determining production of the complexes.

The non-replicating vector employed to elicit the antibodies may be the plasmid vector p82M35B.

The invention also includes a diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

(a) a non-replicating vector as provided herein to produce antibodies specific for the RSV F protein;

(b) isolation means to isolate said RSV F protein specific antibodies;

(c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies; and

(d) identifying means to determine production of the complex.

The present invention is further directed to a method for producing RSV F protein specific polyclonal antibodies comprising the use of the immunization method described herein, and further comprising the step of isolating the RSV F protein specific polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies specific for an F protein of RSV, comprising the steps of:

(a) constructing a non-replicating vector as provided herein;

(b) administering the vector to at least one mouse to produce at least one immunized mouse;

(c) removing B-lymphocytes from the at least one immunized mouse;

(d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(e) cloning the hybridomas;

(f) selecting clones which produce anti-F protein antibody;

(g) culturing the anti-F protein antibody-producing clones; and

(h) isolating anti-F protein monoclonal antibodies.

In this application, the term "RSV F protein" is used to define (1) a full-length mature RSV F protein, such proteins having variations in their amino acid sequences including those naturally occurring in various strains of RSV, (2) a secreted form of RSV F protein lacking a transmembrane region, and (3) functional

analogs of the RSV F protein. In this application, a first protein is a "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution, addition or deletion mutant thereof. Included are RSV F protein fragments that generate antibodies and/or CTLs that specifically react with RSV F protein.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following General Description and Examples with reference to the Figures in which:

Figure 1 illustrates a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus;

Figures 2A, 2B, 2C, 2D and 2E show the nucleotide sequence of the gene encoding the membrane attached form of the F protein of Respiratory Syncytial Virus having its autologous signal peptide (SP) (SEQ ID No: 1) as well as the amino acid sequence of the RSV F protein encoded thereby (SEQ ID No: 2);

Figures 3A, 3B, 3C and 3D show the nucleotide sequence of the gene encoding the secreted form of the RSV F protein having its autologous signal peptide sequence (SP) and lacking the transmembrane region (SEQ ID No: 3) as well as the amino acid sequence of the truncated RSV F protein lacking the transmembrane region encoded thereby (SEQ ID No: 4);

Figures 4A, 4B, 4C and 4D show the construction of plasmid pXL1 containing the gene encoding a secreted form of the RSV F protein lacking the transmembrane region;

Figures 5A, 5B, 5C and 5D show the construction of plasmid pXL2 containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane region and containing the rabbit β-globin Intron II sequence;

Figures 6A, 6B, 6C and 6D show the construction of plasmid pXL3 containing the gene encoding a full length membrane attached form of the RSV F protein;

Figure 7 shows the construction of plasmid pXL4 containing a gene encoding a membrane attached form of the RSV F protein and containing the rabbit β-globin Intron II sequence;

Figure 8 shows the nucleotide sequence for the rabbit β-globin Intron II sequence (SEQ ID No. 5);

Figure 9 shows the lung cytokine expression profile in DNA-immunized mice after RSV challenge;

Figure 10 is a schematic showing the assembly of plasmid p82M35B containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane region, and containing the rabbit β-globin Intron II sequence and the signal peptide sequence HSV I gD;

Figure 11 shows the nucleotide sequence of plasmid VR-1012 (SEQ ID No: 6); and

Figure 12 shows DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequences of the HSV I gD signal peptide sequence, synthesized as a synthetic oligopeptide.

GENERAL DESCRIPTION OF INVENTION

As described above, the present invention relates generally to polynucleotide, including DNA, immunization to obtain protection against infection by respiratory syncytial virus (RSV) and to diagnostic procedures using particular vectors. In the present invention, several recombinant vectors were constructed to contain a nucleotide sequence encoding an RSV F protein. Certain of the vectors described herein also are described in the aforementioned WO 96/04095 and do not form part of this invention but the description of their preparation and use in immunization studies are included herein for completeness and comparison.

The nucleotide sequence of the full length RSV F gene with the sequence encoding the autologous signal peptide is shown in Figure 2 (SEQ ID No: 1). Certain constructs provided herein include the nucleotide sequence encoding the full-length RSV F (SEQ ID No: 2) protein while others include an RSV F gene modified by insertion of termination codons immediately upstream of the transmembrane coding region (see Figure 3, SEQ ID No: 3), to prevent expression of the transmembrane portion of the protein and to produce a secreted or truncated RSV F protein lacking a transmembrane region (SEQ ID No. 4). In addition, certain constructs provided herein in accordance with the present invention include a nucleic acid sequence encoding a heterologous signal peptide sequence rather than

the native signal peptide sequence to provide for enhanced protein expression and increased immunogenicity. Specifically, the signal peptide sequence for HSV I gD is employed for such purpose in the preferred embodiment. However, other heterologous signal peptides may be employed, such as that of human tissue plasminogen activator (TPA).

The nucleotide sequence encoding the RSV F protein is operatively coupled to a promoter sequence for expression of the encoded RSV F protein. The promoter sequence may be the immediately early cytomegalovirus (CMV) promoter. This promoter is described in ref. 13. Any other convenient promoter may be used, including constitutive promoters, such as, Rous Sarcoma Virus LTRs, and inducible promoters, such as metallothionine promoter, and tissue specific promoters.

The vectors provided herein, when administered to an animal, effect *in vivo* RSV F protein expression, as demonstrated by an antibody response in the animal to which it is administered. Such antibodies may be used herein in the detection of RSV protein in a sample, as described in more detail below. When the encoded RSV F protein is in the form of an RSV F protein from which the transmembrane region is absent, such as plasmid pXL1 (Figure 4), the administration of the vector conferred protection in mice and cotton rats to challenge by live RSV virus neutralizing antibody and cell mediated immune responses and an absence of immunopotentiation in immunized animals, as seen from the Examples below.

The recombinant vector also may include a second nucleotide sequence located adjacent the RSV F protein encoding nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* in a host. Such enhancement may be provided by increased *in vivo* expression, for example, by increased mRNA stability, enhanced transcription and/or translation. This additional sequence preferably is located between the promoter sequence and the RSV F protein-encoding sequence.

This enhancement sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing during transcription and translation so that substantially all transcribed mRNA encodes an RSV F protein. Specifically, the rabbit β-globin Intron II sequence shown in Figure 7 (SEQ ID No: 5) may provide such splice sites, as also described in ref. 15.

The construct containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid pXL2 (Figure 5), induced complete protection in mice against challenge with live RSV, as seen in the Examples below. In addition, the construct containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the full-length RSV F protein, i.e. plasmid pXL4 (Figure 7), also conferred protection in mice to challenge with live RSV, as seen from the Examples below. Plasmids pXL1, pXL2, pXL3 and pXL4 all contain the autologous signal peptide sequence and are constructed in accordance with the aforementioned WO 96/04095.

The construct containing the Intron II sequence, CMV promoter, HSV I gD signal peptide peptide encoding sequence and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid p82M35B (Figure 10), in accordance with the invention, induced complete protection in the absence of cardiotoxin pretreatment under conditions where pretreatment with cardiotoxin was required for pXL2 to confer complete protection, as seen from the Examples below.

The vector provided herein may also comprise a third nucleotide sequence encoding a further antigen from RSV, an antigen from at least one other pathogen or at least one immunomodulating agent, such as cytokine. Such vector may contain said third nucleotide sequence in a chimeric or a bicistronic structure. Alternatively, vectors containing the third nucleotide sequence may be separately constructed and coadministered to a host, with the nucleic acid molecule provided herein.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis and treatment of RSV infections. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the RSV F genes and vectors as disclosed herein. The vaccine elicits an immune response in a subject which includes the production of anti-F antibodies. Immunogenic compositions, including vaccines, containing the nucleic acid may be

prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 9324640, ref. 17) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a phospholipid/glycolipid/polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactideco-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain an absorption enhancer.

The RSV F genes and vectors may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intravenously, intradermally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize the RSV F protein and antibodies thereto, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 μg to about 1 mg of the RSV F genes and vectors. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also

belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens to produce immune stimulating complexes (ISCOMS), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as monophoryl lipid A, QS 21 and polyphosphazene.

In particular embodiments of the present invention, the vector comprising a first nucleotide sequence encoding an F protein of RSV may be delivered in conjunction with a targeting molecule to target the vector to selected cells including cells of the immune system.

The polynucleotide may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 10) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice

resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 11) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

2. Immunoassays

The RSV F genes and vectors of the present invention are useful as immunogens for the generation of anti-F antibodies for use in immunoassays, including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art. In ELISA assays, the vector first is administered to a host to generate antibodies specific to the RSV F protein. These RSV F-specific antibodies are immobilized onto a selected surface, for example, a surface capable of binding the antibodies, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed antibodies, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This procedure may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures such as of the order of about 20° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound RSV F specific antibodies, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined.

BIOLOGICAL MATERIALS

Certain plasmids that contain the gene encoding RSV F protein and referred to herein have been deposited with the America Type Culture Collection (ATCC)

located at 10801 University Blvd., Manassas, VA 20110-2209, U.S.A., pursuant to the Budapest Treaty and prior to the filing of this application. Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this or a related United States patent application and all restrictions on access to the deposits will be removed at that time. The deposits will be replaced if the Depository is unable to dispense viable samples. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

Plasmid	ATCC Designation	Date Deposited
pXL1	97167	May 30, 1995
pXL2	97168	May 30, 1995
pXL3	97169	May 30, 1995
pXL4	97170	May 30, 1995
p82M35B	203790	February 23, 1999

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes the construction of vectors containing the RSV F gene.

Figure 1 shows a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus and Figure 2 shows the nucleotide sequence of the gene

encoding the full-length RSV F protein (SEQ ID No: 1) and the deduced amino acid sequence (SEQ ID No: 2). Figure 3 shows the gene encoding the secreted RSV F protein (SEQ ID No: 3) and the deduced amino acid sequence (SEQ ID No: 4).

A set of four plasmid DNA constructs were made (as shown schematically in Figures 4 to 7) in which cDNA encoding the RSV-F was subcloned downstream of the immediate-early promoter, enhancer and intron A sequences of human cytomegalovirus (CMV) and upstream of the bovine growth hormone (BGH) poly-A site. The 1.6 Kb SspI-PstI fragment containing the promoter, enhancer and intron A sequences of CMV Towne strain were initially derived from plasmid pRL43a obtained from Dr. G.S. Hayward of Johns Hopkins University (ref. 20) and subcloned between *Eco*RV and *Pst*I sites of pBluescript 11 SK +/- (Stratagene). For the construction of plasmids expressing the secretory form of the F protein (pXL1 and pXL2 in Figs. 4 and 5), the 1.6 Kb *Eco*RI-*Bam*HI fragment containing the truncated form of the F cDNA originally cloned from a clinical isolate belonging to subgroup A was excised from pRSVF (ref. 18 and WO 93/14207) and subcloned between *Eco*RI and *Bam*HI sites of pSG5 (Strategene, ref. 14). Either the 1.6 kb EcoRI-BamHI fragment or the 2.2 kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The 0.6 kb ClaI-EcoRI fragment derived from pSG5 contained the intron II sequences from rabbit β-globin. Subsequently, the plasmids were digested with HindIII, filled-in with Klenow, and digested with XbaI to yield either a 3.2 or a 3.8 Kb fragment. These fragments were used to replace the 0.8 kb NruI-XbaI fragment containing the CMV promoter in pRc/CMV (Invitrogen), resulting in the final pXL1 and pXL2 constructs, respectively.

For the construction of plasmids expressing the full-length F protein (pXL3 and pXL4 - Figs. 6 and 7), the full length RSV F cDNA was excised as a 1.9 kb EcoRI fragment from a recombinant pBluescript M13-SK (Stratagene) containing the insert (ref. 18 and WO 93/14207) and subcloned at the EcoRI site of pSG5 (Stratagene). Either the 1.9 Kb EcoRI fragment or the 2.5 Kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and

intron A sequences. The rest of the construction for pXL3 and pXL4 was identical to that for pXL1 and pXL2, as described above. Therefore, except for the CMV promoter and intron A sequences, the rest of the vector components in pXL1-4 were derived from plasmid pRc/CMV. Plasmids pXL1 and pXL2 were made to express a truncated/secretory form of the F protein which carried stop codons resulting in a C-terminal deletion of 48 amino acids including the transmembrane (TM) and the C-terminal cytosolic tail as compared to the intact molecule. In contrast, pXL3 and pXL4 were made to express the intact membrane-attached form of the RSV F molecule containing the TM and the cytosolic C-terminal tail. The rationale for the presence of the intron II sequences in pXL2 and pXL4 was that this intron was reported to mediate the correct splicing of RNAs. Since mRNA for the RSV-F has been suspected to have a tendency towards aberrant splicing, the presence of the intron II sequences might help to overcome this. All four plasmid constructs were confirmed by DNA sequencing analysis. Plasmids pXL1, pXL2, pXL3 and pXL4 all contain the autologous signal peptide sequence and are constructed in accordance with the aforementioned WO 96/04095.

Plasmid DNA was purified using plasmid mega kits from Qiagen (Chatsworth, CA, USA) according to the manufacturer's instructions.

Example 2

This Example describes the immunization of mice. Mice are susceptible to infection by RSV as described in ref. 16.

For intramuscular (i.m) immunization, the anterior tibialis anterior muscles of groups of 9 BALB/c mice (male, 6-8 week old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 μg (1 μg/μL in PBS) of pXL1-4, respectively. Five days prior to DNA injection, the muscles were treated with 2 x 50 μL (10 μM in PBS) of cardiotoxin (Latoxan, France). Pretreatment of the muscles with cardiotoxin has been reported to increase DNA uptake and to enhance the subsequent immune responses by the intramuscular route (ref. 24). These animals were similarly boosted a month later. Mice in the control group were immunized with a placebo plasmid containing identical vector backbone sequences without the RSV F gene according to the same schedule. For intradermal (i.d.) immunization,

100 μg of pXL2 (2 μg/μL in PBS) were injected into the skin 1-2 cm distal from the tall base. The animals were similarly boosted a month later.

Seventy-five days after the second immunization, mice were challenged intranasally with $10^{5.4}$ plaque forming units (pfu) of mouse-adapted RSV, A2 subtype (obtained from Dr. P. Wyde, Baylor College of Medicine, Houston, TE, USA). Lungs were aseptically removed 4 days later, weighed and homogenized in 2 mL of complete culture medium. The number of pfu in lung homogenates was determined in duplicates as previously described (ref. 19) using vaccine quality Vero cells. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

Sera obtained from immunized mice were analyzed for anti-RSV F antibody titres (IgG, IgG1 and IgG2a, respectively) by enzyme-linked immunosorbent assay (ELISA) and for RSV-specific plaque-reduction titres. ELISA were performed using 96-well plates coated with immunoaffinity purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. For the measurement of IgG1 and IgG2a antibody titres, the secondary antibodies used were monospecific sheep anti-mouse IgG1 (Serotec, Toronto, Ont., Canada) and rat anti-mouse IgG2a (Zymed, San Francisco, CA, USA) antibodies conjugated to alkaline phosphatase, respectively. Plaque reduction titres were determined according to Prince et al (ref. 19) using vaccine quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of RSV, Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO_2. Vero cells were then infected with the mixture. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV-F monoclonal IgG1 antibody and donkey antimouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes., Mississauga, Ont. Canada). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in the number of plaques. Both ELISA and plaque reduction assays were performed in duplicates and data are expressed as the means of two determinations. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

To examine the induction of RSV-specific CTL following DNA immunization, spleens from 2 immunized mice were removed to prepare single cell suspensions which were pooled. Splenocytes were incubated at 2.5 x 10^6 cells/mL in complete RPMI medium containing 10 U/mL murine interleukin 2 (IL-2) with γ-irradiated (3,000 rads) syngeneic splenocytes (2.5 x 10^6 cells/mL) infected with 1 $TCID_{50}$/cell RSV (Long strain) for 2 hr. The source of murine IL-2 was supernatant of a mouse cell line constitutively secreting a high level of IL-2 obtained from Dr. H. Karasuyama of Basel Institute for Immunology (ref. 20). CTL activity was tested 5 days following the *in vitro* re-stimulation in a standard 4 hr chromium release assay. Target cells were 5 ^{51}Cr-labelled uninfected BALB/c fibroblasts (BC cells) and persistently RSV-infected BCH14 fibroblasts, respectively. Washed responder cells were incubated with 2 x 10^3 target cells at varying effector to target ratios in 200 μL in 96-well V-bottomed tissue-culture plates for 4 hr at 37°C. Spontaneous and total chromium releases were determined by incubating target cells with either medium or 2.5% Triton-X 100 in the absence of responder lymphocytes. Percentage specific chromium release was calculated as (counts-spontaneous counts)/(total counts-spontaneous counts) x 100. Tests were performed in triplicates and data are expressed as the means of three determinations. For antibody blocking studies in CTL assays, the effector cells were incubated for 1 hr with 10 μg/ml final of purified mAb to CD4 (GK1.5) (ref. 21) or mAb against murine CD8 (53-6.7) (ref. 22) before adding chromium labelled BC or BCH4 cells. To determine the effect of anti-class I MHC antibodies on CTL killing, the chromium labelled target cells BC or BCH4 were incubated with 20 μL of culture supernate of hybridoma that secretes a mAb that recognizes K^d and D^d of class I MHC (34-1-2S) (ref. 23) prior to the addition of effector cells.

Example 3

This Example describes the immunogenicity and protection by polynucleotide immunization by the intramuscular route.

To characterize the antibody responses following i.m. DNA administration, immune sera were analyzed for anti-RSV F IgG antibody titre by ELISA and for RSV-specific plaque reduction titre, respectively. All four plasmid constructs were found to be immunogenic. Sera obtained from mice immunized with pXL1-4

demonstrated significant anti-RSV F IgG titres and RSV-specific plaque reduction titres as compared to the placebo group (Table 1 below) ($P<0.0061$ and <0.0001, respectively, Mann-Whitney Test). However, there is no significant difference in either anti-RSV F IgG titre or RSV-specific plaque reduction titre among mice immunized with either pXL1, pXL2, pXL3 or pXL4.

To evaluate the protective ability of pXL1-4 against primary RSV infection of the lower respiratory tract, immunized mice were challenged intranasally with mouse-adapted RSV and viral lung titres post challenge were assessed. All four plasmid constructs were found to protect animals against RSV infection. A significant reduction in the viral lung titre was observed in mice immunized with pXL1-4 as compared to the placebo group ($P<0.0001$, Mann-Whitney Test). However, varying degrees of protection were observed depending on the plasmid. In particular, PXL1 was more protective than pXL3 ($P=0.00109$, Mann-Whitney Test), and pXL4 more than pXL3 ($P=0.00125$), whereas only pXL2 induced complete protection. This conclusion was confirmed by another analysis with number of fully protected mice as end point (Fisher Exact Test). Constructs pXL1, pXL2 or pXL4 conferred a higher degree of protection than pXL3 ($P<0.004$, Fisher Exact Test) which was not more effective than placebo. Only pXL2 conferred full protection in all immunized mice.

The above statistical analysis revealed that pXL1 conferred more significant protection than pXL3. The former expresses the truncated and secretory form and the latter the intact membrane anchored form of the RSV F protein. Furthermore, pXL4 was shown to be more protective than pXL3. The difference between these two constructs is the presence of the intron II sequence in pXL4. Construct pXL2 which expresses the secretory form of the RSV-F in the context of the intron II sequence was the only plasmid that conferred complete protection in all immunized mice in the protocol of Example 2.

Example 4

This Example describes the influence of the route of administration of pXL2 on its immunogenicity and protective ability.

The i.m. and i.d. routes of DNA administration were compared for immunogenicity in terms of anti-RSV F antibody titres and RSV-specific plaque

reduction titres. Analyses of the immune sera (Table 2 below) revealed that the i.d. route of DNA administration was as immunogenic as the i.m. route as judged by anti-RSV F IgG and IgG1 antibody responses as well as RSV-specific plaque reduction titres. However, only the i.m. route induced significant anti-RSV F IgG2a antibody responses, whereas the IgG2a isotype titre was negligible when the i.d. route was used. The i.m. and i.d. routes were also compared with respect to the induction of RSV-specific CTL. Significant RSV-specific CTL activity was detected in mice immunized intramuscularly. In contrast, the cellular response was significantly lower in mice inoculated intradermally (Table 3 below). In spite of these differences, protection against primary RSV infection of the lower respiratory tract was observed in both groups of mice immunized via either route (Table 4 below). The CTL induced by RSV-F DNA are classical CD8+ class I restricted CTL. The target cells, BCH4 fibroblasts express class I MHC only and do not express class II MHC. Further, prior incubation of BCH4 target cells with anti class-I MHC antibodies significantly blocked the lytic activity of RSV-F DNA induced CTL line. While anti-CD8 antibody could partially block lysis of BCH4 cells, antibody to CD4 molecule had no effect at all (Table 5 below). Lack of total blocking by mAb to CD8 could either be due to CTL being CD8 independent (meaning that even though they are CD8+ CTL, their TCR has enough affinity for class I MHC+peptide and it does not require CD8 interaction with the alpha 3 of class I MHC) or the amount of antibody used in these experiments was limiting. There was no detectable lysis of YAC-1 (NK sensitive target) cells (data not shown).

Example 5

This Example describes immunization studies in cotton rats using pXL2.

The immune response of cotton rats to DNA immunization was analyzed by the protocol shown in Table 6 below. On day -5, 40 cotton rats were randomly selected and divided into 8 groups of 5. Cotton rats in groups 1 and 7 were inoculated intramuscularly (i.m.) into the tiberlia anteria (TA) muscles bilaterally with cardiotoxin (1.0 μM). On day -1, the cotton rats in group 8 were inoculated in the TA muscles with bupivacaine (0.25%). On day 0, several animals in each group were bled to determine levels of RSV-specific antibodies in the serum of the test animals prior to administration of vaccines. All of the animals were then inoculated

i.m. or intradermally (i.d.) with 200 μg of plasmid DNA, placebo (non-RSV-specific DNA), 100 median cotton rat infectious doses (CRID50; positive control) of RSV, or of formalin inactivated RSV prepared in Hep-2 tissue culture cells and adjuvanted in alum. Forty-four days later the cotton rats in groups 1 & 7 were reinoculated with cardiotoxin in the TA muscles. Four days later (48 days after priming with vaccine), the animals in group 8 were reinoculated with bupivacains in the TA muscle of the right leg. The next day, (seven weeks after priming with vaccine) all of the animals were bled and all, except those in the group given live RSV, were boosted with the same material and doses used on day 0. 29 days later, each cotton rat was bled and then challenged intranasally (i.n.) with 100 CRID50 RSV A2 grown in Hep-2 tissue culture cells. Four days after this virus challenge (day +88) all of the cotton rats were killed and their lungs removed. One lobe from each set of lungs was fixed in formalin and then processed for histologic evaluation of pulmonary histopathology. The remaining lobes of lung will be assessed for the presence and levels of RSV. Each of the sera collected on days 0, 49 and 78 were tested for RSV-neutralizing activity, anti-RSV fusion activity and RSV-specific ELISA antibody.

The RSV neutralizing titres on day +49 and +78 are shown in Tables 7(a) below and 7(b) below respectively. As can be seen from the results shown in Table 7(a), on day +49 the animals immunized with live RSV and DNA immunization had substantial RSV serum neutralizing titres. The animals immunized with formalin-inactivated RSV had a neutralizing titre equivalent to the placebo group on day +49 but following boosting titres by day +78 had reached 5.8 ($\log_{10}/0.05$). Boosting had no significant effect upon animals immunized with live RSV or by i.m. plasmid immunization.

RSV titres in nasal washes (upper respiratory tract) on day +82 are shown in Table 8 below. RSV titres in the lungs (lower respiratory tract) on day +82 are shown in Table 9 below. All of the vaccines provided protection against lung infection but, under these conditions, only live virus provided total protection against upper respiratory tract infection.

The lungs from the cotton rats were examined histologically for pulmonary histopathology and the results are shown in Table 10 below. With the exception of lung sections obtained from Group 9 which were essentially free of inflammatory

cells or evidence of inflammation, and those from Group 3, which exhibited the maximal pulmonary pathology seen in this study, all of the sections of lung obtained from the other groups looked familiar, i.e. scattered inflammatory cells were present in most fields, and there was some thickening of septae. These are evidence of mild inflammatory diseases. Large numbers of inflammatory cells and other evidence of inflammation were present in sections of lung from Group 3 (in which formalin-inactivated [FI] RSV vaccine was given prior to virus challenge). This result indicated that immunization with plasmid DNA expressing the RSV F protein does not result in pulmonary histopathology different from the placebo, whereas FI-RSV caused more severe pathology.

Example 6

This Example describes the determination of local lung cytokine expression profile in mice imunized with pXL2 after RSV challenge.

Balb/C mice were immunized at 0 and 6 weeks with 100 μg of pXL2, prepared as described in Example 1, and challenged with RSV i.n. at 10 weeks. Control animals were immunized with FI-RSV and live RSV and challenged with RSV according to the same protocol. Four days post viral challenge, lungs were removed from immunized mice and immediately frozen in liquid nitrogen. Total RNA was prepared from lungs homogenized in TRIzol/β-mercaptoethanol by chloroform extraction and isopropanol precipitation. Reverse transcriptase-polymerase chain reaction (RT-PCR) was then carried out on the RNA samples using either IL-4, IL-5 or IFN-γ specific primers from Clone Tech. The amplified products were then liquid-hybridized to cytokine-specific ^{32}P-labeled probes from Clone Tech, resolved on 5% polyacrylamide gels and quantitated by scanning of the radioactive signals in the gels. Three mouse lungs were removed from each treatment group and analyzed for lung cytokine expression for a minimum of two times. The data is presented in Figure 9 and represents the means and standard deviations of these determinations.

As may be seen from the data presented in Figure 9:

1. Immunization with live RSV intranasally (i.n.) resulted in a balanced cytokine profile (IFN-γ, IL-4 and IL-5), whereas that with FI-RSV

intramuscularly (i.m.) resulted in a Th2 predominance (elevated IL-4 and IL-5). These results are similar to what were reported in the literature.

2. Immunization with pXL2 containing the secretary (sec.) form of FI via either the i.m. or intradermal (i.d.) route gave rise to a balanced cytokine profile similar to that with live RSV immunization.

3. The magnitude of the cytokine responses with i.m. and i.d. immunication using pXL2 expressing a secretory form of the protein in significantly higher than that with live RSV immunization.

Example 7

This Example describes the construction of a plasmid vector encoding the RSV F protein and containing the 5' UTR and signal peptide of Herpes Simplex Virus I (HSV I) gD in accordance with the invention.

Plasmid p82M35B was prepared following the scheme shown in Figure 10. Plasmid pVR1012 (Vical) (Figure 11; SEQ ID No: 6) containing the CMV promoter, intron A, and the BGH poly A sequences, was linearized with restriction enzyme Pst I and made blunt ended with T4 DNA polymerase. The rabbit β-globin intron II sequence was retrieved from plasmid pSG5 (Stratagene; ref. 14) by Cla I and Eco RI digestion, and the 0.6 kb fragment was isolated and made blunt ended by treatment with Klenow fragment polymerase. The rabbit β-globin intron II fragment was then ligated to the Pst I/blunt ended VR1012 plasmid (Fig. 10). This vector was then restricted with Eco RV and dephosphorylated.

The secreted form of RSV F was isolated from plasmid pXL2 (Example 1; Fig. 5) by digestion with Sal I, made blunt end by treatment with Klenow fragment polymerase, then restricted with Kpn I to produce a 5' Kpn I, 3' blunt ended fragment. The HSV I gD sequence was synthesized as a synthetic oligonucleotide having the DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequences shown in Figure 12.

The gD oligonucleotide has a 5' blunt end and 3' Kpn I recognition sequence. A three-way ligation was performed with the isolated RSV F fragment, gD oligo and the VR1012 plasmid, to produce plasmid p82M35B (Fig. 10).

Example 8

This Example illustrates the expression and secretion of RSV F protein *in vitro*.

BHK cells were transfected with either p82M35B, prepared as described in Example 7, its counterpart containing the autologous RSV F signal peptide (pXL2), prepared as described in Example 6, or the vector backbone alone (placebo) using Lipofectin (Gtibco/BRL). Forty-eight hours post transfection, supernatant fractions were recovered and subjected to RSV F protein quantification using a F-specific enzyme-linked immunoabsorbent assay (ELISA). Three independent transfection assays were performed for each vector.

ELISAs were performed using one affinity-purified mouse monoclonal anti-RSV F antibody (2 μg/ml) as the capturing reagent and another biotinolated monoclonal anti-RSV F antibody (0.1 μg/ml) as the detection reagent. Horseradish peroxidase-labelled avidin (Pierce) was subsequently used. The RSV F standard protein used was purified from detergent-lysates of cultured virus by immunoaffinity chromatography.

Table 11 (below) shows the results obtained. As seen in Table II, compared to placebo, both p82M35B and pXL2 mediated significant F protein expression/secretion from the BHK cells 48 hours post transfection. Furthermore, a markedly higher level of the F protein was consistently detected in the supernatant fraction of p82M35B-transfected BHK cells than that of pXL2-transfected cells, representing a 5.4-fold improvement over the latter. These results indicate that replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD significantly enhanced F protein expression/secretion *in vitro*.

Example 9

This Example illustrates immunogenicity studies carried out using p82M35B.

Tibialis anterior muscles of BALB/c mice (male, 6 to 8 weeks old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 μg (1 μg/μL in PBS) of p82M35B, pXL2 or the vector backbone alone (placebo). In some groups, 5 days prior to DNA injection, the muscles were treated with 2 x 50 μL (10 μM in

PBS) of cardiotoxin (Latoxan, France), while such pretreatment was omitted in others. The animals were boosted with the same dose of plasmid DNA 6 weeks later. Mice in the positive control group were immunized intranasally (i.n.) with 10^6 plaque forming units (pfu) of a clinical RSV strain of the A2 subtype grown in Hep2 cells (ref. 16).

Antisera obtained from immunized mice were analyzed for anti-RSV F IgG antibody titres using specific ELISA and for RSV-specific plaque-reduction titres. ELISAs were performed using 96-well plates coated with immunoaffinity-purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. Plaque reduction titres were determined according to Prince et al. (ref. 19) using vaccine-quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of the RSV Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO_2 and the mixtures were used to infect Vero cells. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV F monoclonal IgG1 antibody and donkey anti-mouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes. Mississauga, Ont.). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in plaque number. Both ELISAs and plaque reduction assays were performed in duplicate and data are expressed as the means of two determinations.

The results of these studies are set forth in Table 12 below. For the induction of serum antibody responses (Table 12), p82M35B is effective without the need of cardiotoxin pretreatment under the DNA dose and immunization regimen used, resulting in anti-F IgG titre of 7.2 ± 1.1 ($\log_2$ titre/100) and RSV-specific plaque reduction titre of 11.8 ± 0.9 ($\log_2$) after two immunizations. In contrast, the antibody titres elicited by pXL2 in the absence of the cardiotoxin pretreatment were significantly lower (IgG titre of 2.9 ± 2.3 and plaque reduction titre of 8.2 ± 1.9). However, serum antibody responses elicited by pXL2 were significantly improved with the cardiotoxin pretreatment step (IgG titre of 7.4 ± 1.1 and plaque reduction titre of 10.5 ± 0.8). The placebo was unable to elicit a detectable serum antibody response in the absence or presence of the cardiotoxin pretreatment step.

This trend was extendible to results of the protection study (Table 12). Vector p82M35B conferred full protection against RSV infection of lungs in the absence of the cardiotoxin pretreatment. In contrast, pXL2 only conferred partial protection under the same conditions. However, full protection was achieved with the pXL2 vector when cardiotoxin pretreatment step was included in the immunization regimen. No protection was observed with the placebo with or without the cardiotoxin pretreatment step.

These results show the replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD resulted in significant enhancement in not only F protein expression/secretion assessed *in vitro* (Example 8), but also immunogenicity to the F protein as well as protective ability against RSV infection assessed in the mouse model.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides certain novel vectors containing genes encoding an RSV F proteins, methods of immunization using such vectors and methods of diagnosis using such vectors. Modifications are possible within the scope of this invention.

Table 1: Immunogenic and Protective Abilities of pXL1-4 Mice via the i.m. Route

Plasmid DNA Immunogen	No. Mice	Mean Anti-RSV F ELISA Titre(IgG)* (Log_2/100±SD)	Mean Plaque Reduction Titre* (Log_4±SD)	Post RSV Challenge: Mean Virus Lung Titre# (pfu/g lung) (Log_{10} ±SD)	Post RSV Challenge: No. Fully Protected Mice**
pXL1	8	3.00±1.85	3.74±0.98	0.72±0.99	5
pXL2	9	5.78±1.72	4.82±0.51	0.00±0.00	9
pXL3	8	3.75±2.05	4.59±1.16	2.77±0.72	0
pXL4	9	5.44±1.13	5.18±0.43	0.66±1.00	6
Placebo**	12	0.58±2.89	0.18±0.62	3.92±0.27	0

* These sets of data from sera obtained 1 week prior to the viral challenge

\# Detection sensitivity of the assay was $10^{1.96}$ pfu/g lung.

** The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 2. Immunogenicity of pXL2 in Mice*

Route	No. Mice	Mean Anti-RSV F ELISA Titre (Log_2/100 + SD)			Mean Plaque Reduction Titre (Log_4 ± SD)
		IgG	IgG1	IgG2a	
i.m	9	7.63±0.92	4.25±1.91	4.38±1.92	4.18±0.88
i.d.	7	7.00±1.00	5.00±1.00	0.14±0.38	3.65±0.59
Placebo(i.m.)	9	0.50±0.51	0.00±0.00	0.00±0.00	0.18±0.50

* These sets of data are from sera obtained 1 week prior to the viral challenge.

Table 3. Induction of RSV-specific CTL Following DNA Immunization*

Route	E:T Ratio	% Specific Lysis	
		BC	BCH4
i.m.	200:1	23.3	100.6
	100:1	17.0	62.4
	50:1	19.9	64.1
	25:1	22.3	46.4
i.d.	100:1	20.9	26.1
	50:1	21.7	19.1
	25:1	7.1	7.0
	12.5:1	2.8	2.3

* These set of data were obtained from immunized mice immediately prior to RSV challenge.

Table 4. Immunoprotective Ability of pXL2 in Mice

Route	No. Mice	Post RSV Challenge	
		Mean Virus Lung Titre* (pfu/g lung)	No. Fully Protected Mice#
i.m.	8	0.00±0.00	8
i.d.	7	0.43±1.13	6
Placebo (i.m.)	9	4.30±022	0

* Detection sensitivity of the assay was $10^{1.69}$ pfu/g lung.

\# The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 5. RSV specific CTL included by i.m. DNA immunization are class I restricted CTL

E:T Ratio	BCH4	BCH4+anti-CD4	BCH4+anti-CD8	BCH4+anti-class I MHC
100:1	52.03	54.3	39.4	8.6
50:1	44.4	47.2	27.4	6.2
25:1	28.6	26.3	14.8	1
12.5:1	18.2	15	8	-2.7

Table 6

Group	Antigen	RSV-specific dose	Inoc. route	Pretreatment/ Adjuvant	Day 0	Day 49	Day 78	Day 88
1	Placebo	0	I.M.	Cardiotoxin	Prebleed, several cotton rats per group; prime all animals	Bleed all animals; boost all except those in group 2	Challenge with RSV A2 I.N. after bleeding all	Harv. animals and do histologic evaluation, pulmonary virus titers, antibodies
2	Live RSV	100 CRID50	I.N.	None				
3	FI-RSV		I.M.	Alum				
5	pXL2	200 μg	I.M.	None				
6	pXL2	200 μg	I.D.	None				
7	pXL2	200 μg	I.M.	Cardiotoxin				
8	pXL2	200 μg	I.M.	Bupivacaine				

Table 7(a). RSV Serum Neutralizing Titers on Day 49

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer (log_2/0.05 ml) in CR no.				Mean titer log2/ 0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4	4	2	2	2.75	1.0
2	Live RSV	100 CRID50	I.N.	9	9	9	9	9	0.0
3	FI-RSV		I.M.	0	4	2	2	2.0	1.0
5	pXL2	200 µg	I.M.	8	8	8	7	8.0	0.8
6	pXL2	200 µg	I.D.	5	2	5	5	4.3	1.5
7	pXL2	200 µg	I.M.	8	8	9	9	8.5	0.6
8	pXL2	200 µg	I.M.	8	8	6	6	7.3	1.5

Table 7(b). RSV Serum Neutralizing Titers on Day 78

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer (log_2/0.05 ml) in CR no.				Mean titer log_2/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	9	I.M.	3	2	4	Died	3.0	1.0
2	Live RSV	100 CRID50	I.N.	8	9	9	9	8.5	0.6
3	FI-RSV		I.M.	8	4	6	5	5.8	1.7
5	pXL2	200 µg	I.M.	8	8	8	8	7.8	0.5
6	pXL2	200 µg	I.D.	9	8	6	Died	6.7	1.2
7	pXL2	200 µg	I.M.	8	9	8	9	8.7	0.6
8	pXL2	200 µg	I.M.	8	7	8	9	8.3	1.0

Table 8. RSV Titers in Nasal Washes on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer (log_{10}/0.05 ml) in cotton rat no.				Mean titer log_{10}/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3.3	3.3	3.3	Died	3.3	0.1
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV		I.M.	0	0	2.8	0	0.7	1.4
5	pXL2	200 µg	I.M.	3.3	2.3	3.3	2.3	2.8	0.6
6	pXL2	200 µg	I.D.	N.D.	N.D.	N.D.	Died	N.D.	N.D.
7	pXL2	200 µg	I.M.	2.3	0	0	3.2	1.4	1.6
8	pXL2	200 µg	I.M.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. = non-determined

Table 9. Titers in Lungs on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer (log_{10}/g lung) in cotton rat no.				Mean titer log_{10}/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3.7	4.2	3.7	Died	4.2	0.5
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV	10^5 PFU	I.M.	0	0	0	0	0.0	0.0
5	pXL2	200 µg	I.M.	0	2.2	0	0	0.6	1.1
6	pXL2	200 µg	I.D.	0	2.2	2.7	3.2	2.0	N.D.
7	pXL2	200 µg	I.M.	0	0	0	0	0.0	0.0
8	pXL2	200 µg	I.M.	0	0	0	0	0.0	N.D.

N.D. = non-determined

Table 10. Summary of Histopathology Results Seen in Sections of Cotton Rat Lung.

Group	Treatment	Major Observations & Comments
1.	Placebo + RSV	Scattered individual and groups of macrophages and polymorphonuclear neutrophiles (PMN) in all fields. Overt thickening of septae. Occasional pyknotic cells seen. Overall: mild to moderate inflammation.
2.	Live RSV	Isolated macrophages seen in most fields. Scattered PMN. Overall: minimal inflammation
3.	FI-RSV + RSV	Virtually every field contains numerous mononuclear cells & PMN. Pyknotic cells and debris common. Thickened septae. Evidence of exacerbated disease.
5.	Plasmid + RSV	Isolated macrophages seen in most fields. Occasional PMN seen. Very similar to live virus group.
6.	Plasmid i.d. + RSV	Isolated macrophages seen in most fields. Occasional PMN seen.
7.	Plasmid + CT + RSV	Isolated mononuclear cells and PMN seen in most fields.
8.	Plasmid + Biv + RSV	Scattered mononuclear cells and PMN seen in most fields.
9.	Normal CR Lung	Few leukocytes evidence. Airy, open appearance. Thin septae.

CT = carditoxin
Biv = bupivacaine

Table 11. Expression/Secretion of the RSV F protein from BHK cells (48 hr post transfection)

Plasmid Construct	F Protein Secretion (mean ± S.D.) (ng/mL)	Magnitude of Improvement
Placebo	0.0 ± 0.0	
p82M35B	32.1 ± 2.06	5.4 x (over pXL2)
pXL2	5.9 ± 0.6	

Table 12. Immunoprotective Ability of DNA-F in BALB/c Mice

Immunogen	Anti-F IgG Titre Log2 (titre/100) 10 weeks	RSV-Specific Plaque Reduction Titre (Log_2 titre)	Mean Virus Lung Titre* (pfu/g lung) (Log_2 10 ± SD)	No. Fully protected # / No. Immunized
Placebo (i.m.)	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.5	0/6
p82M35B (i.m.)	7.2 ± 1.1	11.8 ± 0.9	0.0 ± 0.0	6/6
pXL2 (i.m.)	2.9 ± 2.3	8.2 ± 1.9	2.9 ± 1.7	1/6
pXL2 + cardiotoxin	7.4 ± 1.1	10.5 ± 0.8	0.0 ± 0.0	6/6
RSV (i.n.)	8.5 ± 2.7	12.4 ± 0.7	0.0 ± 0.0	6/6

* Sensitivity of assay: $10^{1.69}$ pfu/g lung.

The term, fully protected mice, refers to animals with no detectable RSV in the lungs 4 days post viral challenge.

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CLAIMS

1. A vector for *in vivo* administration to a host, comprising:

a nucleotide sequence encoding an RSV F protein lacking an autologous RSV F signal peptide sequence and including a nucleotide sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein in the host; and

a promoter sequence operatively coupled to the nucleotide sequence for expression of said RSV F protein in the host.

2. The vector of claim 1 wherein said nucleotide sequence encoding a heterologous signal peptide encodes Herpes Simplex Virus I (HSV I) gD.

3. The vector of claim 1 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

4. The vector of claim 1 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

5. The vector of claim 1 further including a second nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

6. The vector of claim 5 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing.

7. The vector of claim 6 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.

8. The vector of claim 7 wherein said second nucleotide sequence is that of rabbit β-globin intron II.

9. The vector of claim 1 which is a plasmid vector.

10. The vector of claim 1 which is plasmid p82M35B as shown in Figure 10.

11. An immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a vector as claimed in claim 1 and a pharmaceutically-acceptable carrier therefor.

12. A method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to said host an effective amount of an immunogenic composition of claim 11.

13. A method of using a nucleotide sequence encoding an RSV F protein lacking an autologous RSV F signal peptide sequence and including a heterologous signal peptide which enhances the level of expression of RSV F protein, which comprises:

isolating a gene encoding an RSV F protein having an autologous RSV F signal peptide sequence;

substituting a nucleotide sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein for the nucleotide sequence encoding the autologous RSV F signal peptide sequence to form said nucleotide sequence;

operatively linking said nucleotide sequence to at least one control sequence to produce a vector, said control sequence directing expression of said RSV F protein when said vector is introduced into a host to produce an immune response to said RSV F protein; and

introducing said vector into the host.

14. The method of claim 13 wherein said nucleotide sequence encoding a heterologous signal peptide encodes Herpes Simplex Virus I (HSV I) gD.

15. The method of claim 13 wherein said nucleotide sequence encoding an RSV F protein encodes an RSV F protein lacking the transmembrane region.

16. The method of claim 15 wherein said at least one control sequence comprises the immediate early cytomegalovirus promoter.

17. The method of claim 16 including the step of:

operatively linking said nucloetide sequence to an immunoprotective enhancing sequence to produce an enhanced immunoprotection to said RSV F protein in said host.

18. The method of claim 17 wherein said immunoprotective enhancing sequence is introduced into said vector between said control sequence and said nucleotide sequence.

19. The method of claim 18 wherein said immunoprotection enhancing sequence comprises a pair of splice sites to prevent aberrant mRNA splicing.

20. The method of claim 19 wherein said immunoprotection enhancing sequence is that of rabbit β-globin intron II.

21. The method of claim 13 wherein said nucleotide sequence is contained within the plasmid vector p82M35B.

22. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein having an autologous RSV F signal peptide sequence;

substituting a nucleotide sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein for the nucleotide sequence encoding the autologous RSV F signal peptide sequence to form a second nucleotide sequence;

operatively linking said second nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of said RSV F protein when introduced into a host to produce an immune response to said RSV F protein; and

formulating said vector as a vaccine for *in vivo* administration.

23. The method of claim 22 wherein said non-replicating vector is the plasmid vector p82M35B.

24. A vaccine produced by the method of claim 22.

RESTRICTION MAP OF THE RSV F GENE
5'
3'
bp
500
1000
1500
AccI
EarI
PpuMI
BclI
MamI
NialV
NsiI
BspHI

FIG.1

FIG.2A.

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

SP

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5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
   ATGGAGTTGCCAATCCTCAAAGCAAATGCAATTACCACAATCCTCGCTGCAGTCACATTT
   TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTTAGGAGCGACGTCAGTGTAAA
        10        20        30        40        50        60

   CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
   TGCTTTGCTTCTAGTCAAAACATCACTGAAGAATTTTATCAATCAACATGCAGTGCAGTT
   ACGAAACGAAGATCAGTTTTGTAGTGACTTCTTAAAATAGTTAGTTGTACGTCACGTCAA
        70        80        90       100       110       120

   SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
   AGCAAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATACTAGTGTTATAACTATAGAA
   TCGTTTCCGATAGAATCACGAGATTCTTGACCAACCATATGATCACAATATTGATATCTT
        130       140       150       160       170       180

   LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
   TTAAGTAATATCAAGGAAAATAAGTGTAATGGAACAGATGCTAAGGTAAAATTGATGAAA
   AATTCATTATAGTTCCTTTTATTCACATTACCTTGTCTACGATTCCATTTTAACTACTTT
        190       200       210       220       230       240

   GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU LEU MET GLN SER THR
   CAAGAATTAGATAAATATAAAAATGCTGTAACAGAATTGCAGTTGCTCATGCAAAGCACA
   GTTCTTAATCTATTTATATTTTTACGACATTGTCTTAACGTCAACGAGTACGTTTCGTGT
        250       260       270       280       290       300

   PRO ALA ALA ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
   CCAGCAGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATTATACACTCAAC
   GGTCGTCGTTTGTTAGCTCGGTCTTCTTCTTGATGGTTCCAAATACTTAATATGTGAGTTG
        310       320       330       340       350       360
```

FIG.2B.

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG PHE LEU GLY PHE
AATACCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAA8AAGATTTCTTGGTTTT
TTATGGTTTTTTTTGGTTACATTGTAATTCGTTCTTTTCCTTTTCTTCTAAAGAACCAAAA
370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTA
AACAATCCACAACCTAGACGTTAGCGGTCACCGTAACGACATAGATTCCAGGACGTGAAT
430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
GAAGGAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAACAAGGCCGTAGTCAGC
CTTCCTCTTCACTTGTTCTAGTTTTCACGAGATGATAGGTGTTTGTTCCGGCATCAGTCG
490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
TTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGAT
AATAGTTTACCTCAATCACAGAATTGGTCGTTTCACAATCTGGAGTTTTTGATATATCTA
550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL
AAACAATTGTTACCTATTGTGAATAAGCAAAGCTGCAGAATATCAAATATAGAAACTGTG
TTTGTTAACAATGGATAACACTTATTCGTTTCGACGTCTTATAGTTTATATCTTTGACAC
610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU GLU ILE THR ARG GLU PHE SER VAL ASN
ATAGAGTTCCAACAAAAGAACAACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
TATCTCAAGGTTGTTTTCTTGTTGTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA
670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU SER LEU
GCAGGTGTAACTACACCTGTAAGCACTTACATGTTAACTAATAGTGAATTATTGTCATTA
CGTCCACATTGATGTGGACATTCGTGAATGTACAATTGATTATCACTTAATTACAGTAAT
730 740 750 760 770 780

FIG.2C.

```
ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
ATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTTCAAATA
TAGTTACTATACGGATATTGTTTACTAGTCTTTTTCAATTACAGGTTGTTACAAGTTTAT
       790       800       810       820       830       840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
GTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTA
CAATCTGTCGTTTCAATGAGATAGTACAGGTATTATTTTCTCCTTCAGAATCGTATACAT
       850       860       870       880       890       900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
GTACAATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACACATCCCCT
CATGTTAATGGTGATATACCACACTATCTATGTGGAACACCTTTAATGTGTGTAGGGGA
       910       920       930       940       950       960

LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
CTATGTACAACCAACACAAAGAGAAGGGTCAAACATCTGTTTACAAGAACTGACAGAGGA
GATACATGTTGGTTGTGTTTTCTTCCCAGTTTGTAGACAAATTGTTCTTGACTGTCTCCT
       970       980       990      1000      1010      1020

TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
TGGTACTGTGACAATGCAGGATCAGTATCTTTTCTTCCCACAAGCTGAAACATGTAAAGTT
ACCATGACACTGTTACGTCCTAGTCATAGAAAGAAGGGTGTTCGACTTTGTACATTTCAA
      1030      1040      1050      1060      1070      1080

GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
CAATCGAATCGAGTATTTTGTGACACAATGAACAGTTTAACATTACCAAGTGAAGTAAAT
GTTAGCTTAGCTCATAAAACACTGTGTTACTTGTCAAATTGTAATGGTTCACTTCATTTA
      1090      1100      1110      1120      1130      1140

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
CTCTGCAATGTTGACATATTCAATCCCAAATATGATTGTAAAATTATGACTTCAAAAACA
GAGACGTTACAACTGTATAAGTTAGGGTTTATACTAACATTTTTAATACTGAAGTTTTTGT
      1150      1160      1170      1180      1190      1200
```

```
ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATCACATCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTTGA
         1210      1220      1230      1240      1250      1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAAAATCGTGGAATCATAAAGACATTTTCTAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCACACTA
         1270      1280      1290      1300      1310      1320

TYR VAL SER ASN LYS GLY [VAL] ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAAATAAAGGGGTGGACACTGTGTCTGTAGGTAACACATTATATTATGTAAAT
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCATTGTGTAATATAATACATTTA
         1330      1340      1350      1360      1370      1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAGTCTCTATGTAAAAGGTGAACCAATAATAAATTTCTATGACCCA
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTATTTAAAGATACTGGGT
         1390      1400      1410      1420      1430      1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAATGAGAAGATTAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG
         1450      1460      1470      1480      1490      1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
         1510      1520      1530      1540      1550      1560
                  <-------------------- TM ------------------------

SER THR THR ASN ILE MET ILE THR THR ILE ILE ILE GLU ILE ILE VAL ILE LEU LEU SER
TCAACCACAAATATCATGATAACTACTATAATTATAGAGATTATAGTAATATTGTTATCA
AGTTGGTGTTTATAGTACTATTGATGATATTAATATCTCTAATATCATTATAACAATAGT
         1570      1580      1590      1600      1610      1620
```

FIG.2D.

```
                                                                  LEU  ILE  ALA  VAL  GLY  LEU  LEU  LEU  TYR  CYS  LYS  ALA  ARG  SER  THR  PRO  VAL  THR  LEU  SER
                                                                  TTAATTGCTGTTGGACTGCTCCTATACTGTAAGGCCAGAAGCACACCAGTCACACTAAGC
                                                                  AATTAACGACAACCTGACGAGGATATGACATTCCGGTCTTCGTGTGGTCAGTGTGATTCG
                                                                                  1640                 1650                 1660                 1670                 1680

LYS,  ASP  GLN  LEU  SER  GLY  ILE  ASN  ASN  ILE  ALA  PHE  SER  ASN
AAGGATCAACTGAGTGGTATAAATAATATTGCATTTAGTAACTGAATAAAAATAGCACCT
TTCCTAGTTGACTCACCATATTTATTATAACGTAAATCATTGACTTATTTTTATCGTGGA
          1690                 1700                 1710                 1720                 1730                 1740

AATCATGTTCTTACAATGGTTACTATCTGCTCATAGACAACCCATCTATCATTGGATTT
TTAGTACAAGAATGTTACCAATGATAGACGAGTATCTGTTGGGTAGATAGTAACCTAAA
          1750                 1760                 1770                 1780                 1790                 1800

TCTTAAAATCTGAACTTCATCGAAACTCTTATCTATAAACCATCTCACTTACACTATTTA
AGAATTTAGACTTGAAGTAGCTTTGAGAATAGATATTTGGTAGAGTGAATGTGATAAAT
          1810                 1820                 1830                 1840                 1850                 1860

AGTAGATTCCTAGTTTATAGTTATAT  3'
TCATCTAAGGATCAAATATCAATATA
          1870                 1880

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA)
STRAND SENSE IN THE 5' TO 3' DIRECTION.THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM)
ANCHOR DOMAIN ARE UNDERLINED.THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW
(↑).
```

FIG. 2e

FIG. 3A.

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

SP

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
ATGGAGTTGCCAATCCTCAAAGCAAATGCAATTACCACAATCCTCGCTGCAGTCACATTT
TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTTAGGAGCGACGTCAGTGTAAA
10 20 30 40 50 60

CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
TGCTTTGCTTCTAGTCAAACATCACTGAAGAATTTTATCAATCAACATGCAGTCAGTTT
ACGAAACGAAGATCAGTTTTGTAGTGACTTCTTAAAATAGTTAGTTGTACGTCAGTCAA
70 80 90 100 110 120

SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
AGCAAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATACTAGTGTTATAACTATAGAA
TCGTTTCCGATAGAATCACGAGATTCTTGACCAACCATATGATCACAATATTGATATCTT
130 140 150 160 170 180

LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
TTAAGTAATATCAAGGAAAATAAGTGTAATGGAACAGATGCTAAGGTAAAATTGATGAAA
AATTCATTATAGTTCCTTTTATTCACATTACCTTGTCTACGATTCCATTTTAACTACTTTT
190 200 210 220 230 240

GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU LEU MET GLN SER THR
CAAGAATTAGATAAATATAAAAATGCTGTAACAGAATTGCAGTTGCTCATGCAAAGCACA
GTTCTTAATCTATTTATATTTTTACGACATTGTCTTAACGTCAACGAGTACGTTTCGTGT
250 260 270 280 290 300

PRO ALA ALA ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
CCAGCAGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATTATACACTCAAC
GGTCGTCGTTTGTTAGCTCGGTCTTCTCTTGATGGTTCCAAATACTTAATATGTGAGTTG
310 320 330 340 350 360

FIG.3B.

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG↓PHE LEU GLY PHE
AATACCAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAAGGAAA8AAGATTTCTTGGTTTT
TTATGGTTTTTTTGGTTACATTGTAATTCGTTCTTTTCCTTTTCTTCTAAAGAACCAAAA
370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTA
AACAATCCACAACCTAGACGTTAGCGGTCACCGTAACGACATAGATTCCAGGACGTGAAT
430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
GAAGGAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAACAAGGCCGTAGTCAGC
CTTCCTCTTCACTTGTTCTAGTTTTCACGAGATGATAGGTGTTTGTTCCGGCATCAGTCG
490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
TTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGAT
AATAGTTTACCTCAATCACAGAATTGGTCGTTTCACAATCTGGAGTTTTTGATATATCTA
550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL
AAACAATTGTTACCTATTGTGAATAAGCAAAGCTGCAGAATATCAAATATAGAAACTGTG
TTTGTTAACAATGGATAACACTTATTCGTTTCGACGTCTTATAGTTTATATCTTTGACAC
610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN
ATAGAGTTCCAACAAAAGAACAACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
TATCTCAAGGTTGTTTTCTTGTTGTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA
670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU
GCAGGTGTAACTACACCTGTAAGCACTTACATGTTAACTAATAGTGAATTATTGTCATTA
CGTCCACATTGATGTGGACATTCGTTGAATGTACAATTGATTATCACTTAATAACAGTAAT
730 740 750 760 770 780

FIG.3C.

```
ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
ATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTTCAAATA
TAGTTACTATACGGATATTGTTTACTAGTCTTTTTCAATTACAGGTTGTTACAAGTTTAT
         790       800       810       820       830       840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
GTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTTAGCATATGTA
CAATCTGTCGTTTCAATGAGATAGTACAGGTATTATTTTCTCCTTCAGAATCGTATACAT
         850       860       870       880       890       900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
GTACAATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACATCCCCT
CATGTTAATGGTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTAGGGGA
         910       920       930       940       950       960

LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
CTATGTACAACCACACAAAGAGGTCAAACATCTGTTTAACAAGAACTGACAGAGGA
GATACATGTTGGTTGTGTTTCTTCCCAGTTTGTAGACAAATTGTTCTTGACTGTCTCCT
         970       980       990      1000      1010      1020

TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
TGGTACTGTGACAATGCAGGATCAGTATCTTTCTTCCCACAAGCTGAAACATGTAAAGTT
ACCATGACACTGTTACGTCCTAGTCATAGAAAGAAGGGTGTTCGACTTTGTACATTTCAA
        1030      1040      1050      1060      1070      1080

GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
CAATCGATCCGAGTATTTTGTGACACAATGAACAGTTTAACATTACCAAGTGAAGTAAAT
GTTAGCTTAGCTCATAAAACACTGTGTTACTTGTCAAATTGTAATGGTTCACTTCATTTA
        1090      1100      1110      1120      1130      1140

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
CTCTGCAATGTTGACATATTCAATCCCAAATATGATTGTAAAATTATGACTTCAAAACA
GAGACGTTACAACTGTATAAGTTTAGGGTTTATACTAACATTTAATACTGAAGTTTTGT
        1150      1160      1170      1180      1190      1200
```

FIG.3D.

```
ASP VAL SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATCACATCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTTGA
      1210       1220       1230       1240       1250       1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAAAATCGTGGAATCATAAAGACATTTTCTAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCACACTA
      1270       1280       1290       1300       1310       1320

TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAAATAAAGGGGTGGACACTGTGTCTGTAGGTAACACATTATATTATGTAAAT
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCATTGTGTAATATAATACATTTA
      1330       1340       1350       1360       1370       1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAGTCTCTATGTAAAAGGTGAACCAATAAJAAATTTCTATGACCA
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTTATTTAAAGATACTGGGT
      1390       1400       1410       1420       1430       1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAATGAGAAGATTAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG
      1450       1460       1470       1480       1490       1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
      1510       1520       1530       1540       1550       1560

SER THR THR ASN ILE MET Thr Stop Stop Stop Bam HI
TCAACCACAAATATCATGACTTGATAATGAGGATCCC
AGTTGGTGTTTATAGTACTGAACTATTACTCCTAGG
      1570
```

HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
EcoRV / PstI
SspI / PstI
SspI
Pcmv + IA
PstI
pRL43a

FIG.4A

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
EcoRI / BamHI / Klenow
BamHI
EcoRI
ClaI
Intron II
Trun. F
pSG5

FIG.4B

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
Trun. F
XbaI
HindIII / Klenow / XbaI
NruI / XbaI
NruI
Pcmv
XbaI
BGH pA
pRc / CMV
SV40 ori
Amp
SV40 pA
NEO

FIG.4C

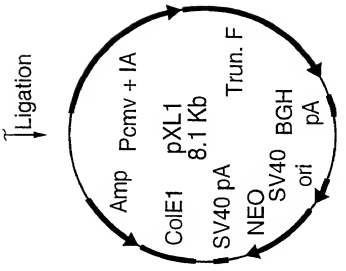
Ligation
Amp
Pcmv + IA
pXL1
8.1 Kb
Trun. F
BGH
pA
SV40
ori
NEO
SV40 pA
ColE1

FIG.4D

HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
EcoRV / PstI
SspI / PstI
SspI
Pcmv + IA
PstI
pRL43a

FIG.5A

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
Intron II
EcoRI
Trun. F
pSG5
BamHI
ClaI / BamHI / Klenow

FIG.5B

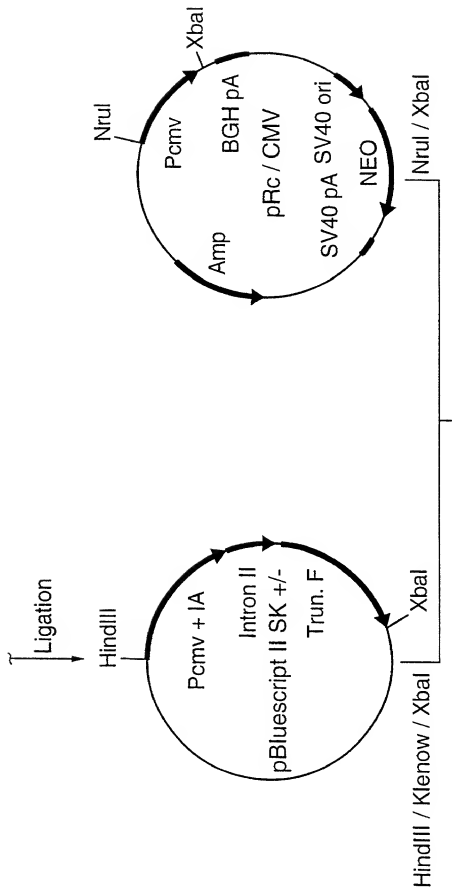
Ligation
HindIII
Pcmv + IA
Intron II
pBluescript II SK +/-
Trun. F
XbaI
HindIII / Klenow / XbaI
NruI / XbaI
NruI
Pcmv
XbaI
BGH pA
pRc / CMV
SV40 ori
NEO
SV40 pA
Amp

FIG.5C

09/914621

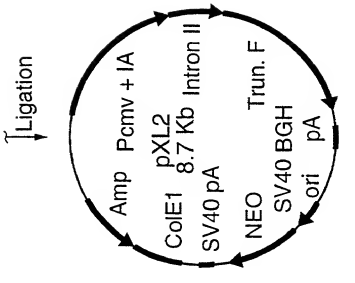

FIG.5D

HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
EcoRV / PstI
SspI / PstI
SspI
Pcmv + IA
PstI
pRL43a

FIG.6A

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
EcoRI /
Klenow
ClaI
Intron II
Intact F
pSG5
EcoRI
EcoR
BamHI

FIG.6B

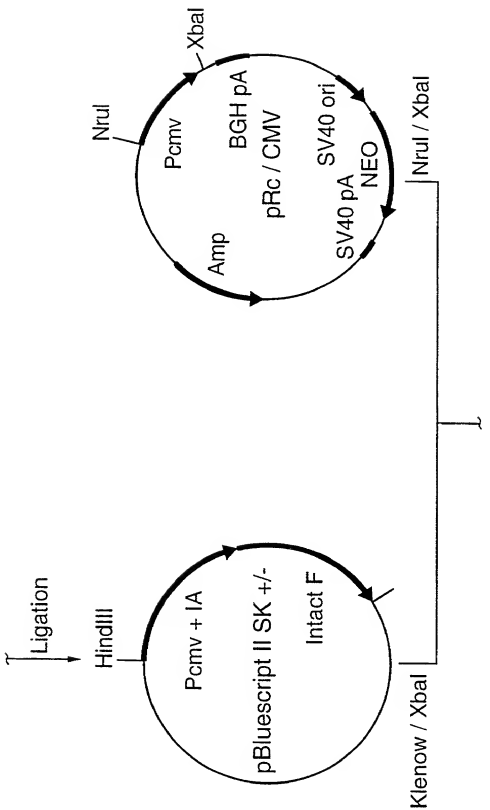
Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
Intact F
HindIII / Klenow / XbaI
NruI / XbaI
NruI
Pcmv
XbaI
BGH pA
pRc / CMV
SV40 ori
NEO
SV40 pA
Amp

FIG.6C

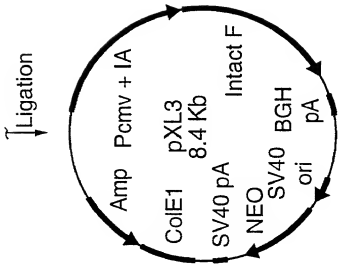
Ligation
Amp
Pcmv + IA
pXL3
8.4 Kb
Intact F
ColE1
SV40 pA
NEO
SV40
BGH
ori
pA

FIG.6D

09/914621

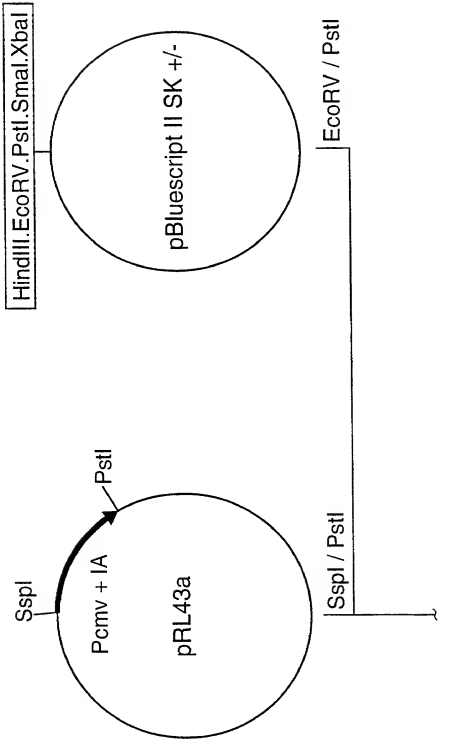

FIG.7A

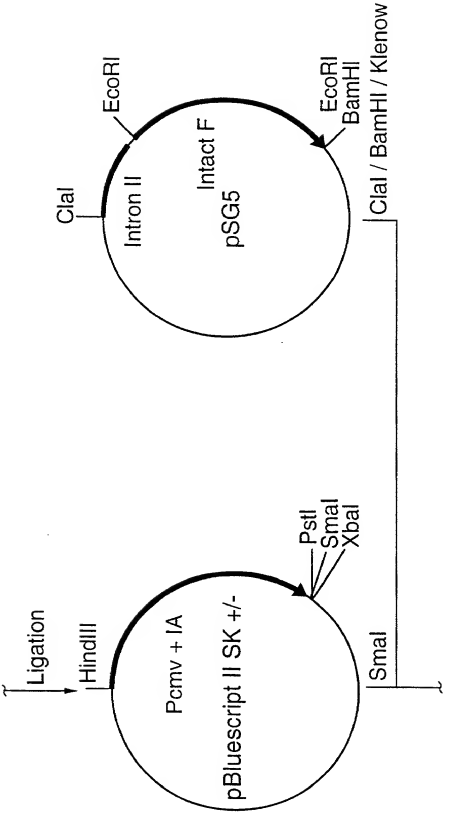
Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
Intron II
EcoRI
Intact F
pSG5
EcoRI
BamHI
ClaI / BamHI / Klenow

FIG.7B

Ligation
HindIII
Pcmv + IA
Intron II
pBluescript II SK +/-
Intact F
XbaI
HindIII / Klenow / XbaI
NruI / XbaI
NruI
Pcmv
XbaI
BGH pA
pRc / CMV
SV40 ori
NEO
SV40 pA
Amp

FIG.7C

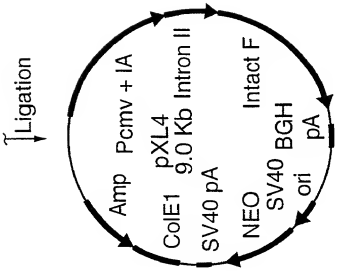

FIG.7D

FIG.8

```
                                                                GTGAGT
401 TTGGGGACCC TTGATTGTTC TTTCTTTTTC GCTAATTGTAA AATTCATGTT
451 ATATGGAGGG GGCAAAGTTT TCAGGGTGTT GTTTAGAATG GGAAGATGTC
501 CCTTGTATCA CCATGGACCC TCATGATAAT TTTGTTTCTT TCACTTTCTA
551 CTCTGTTGAC AACCATTGTC TCCTCTTATT TTCTTTTCAT TTTCTGTAAC
601 TTTTTCGTTA AACTTTAGCT TGCATTTGTA ACGAATTTTT AAATTCACTT
651 TTGTTTATTT GTCAGATTGT AAGTACTTTC TCTAATCACT TTTTTTTCAA
701 GGCAATCAGG GTATATTATA TTGTACTTCA GCACAGTTTT AGAGAACAAT
751 TGTTATAATT AAATGATAAG GTAGAATATT TCTGCATATA AATTCTGGCT
801 GGCGTGGAAA TATTCTTATT GGTAGAAACA ACTACATCCT GGTCATCATC
851 CTGCCTTTCT CTTTATGGTT ACAATGATAT ACACTGTTTG AGATGAGGAT
901 AAAATACTCT GAGTCCAAAC CGGGCCCCTC TGCTAACCAT GTTCATGCCT
951 TCTTCTTTTT CCTACAG
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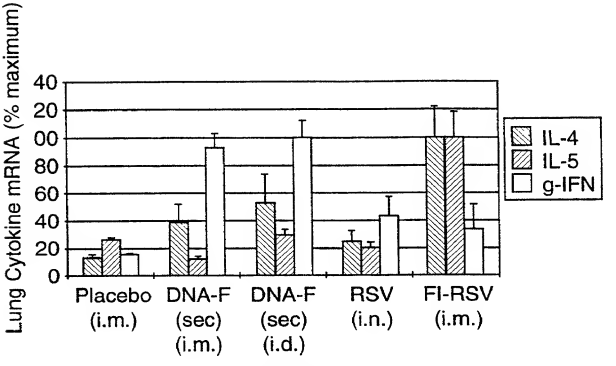

FIG.9

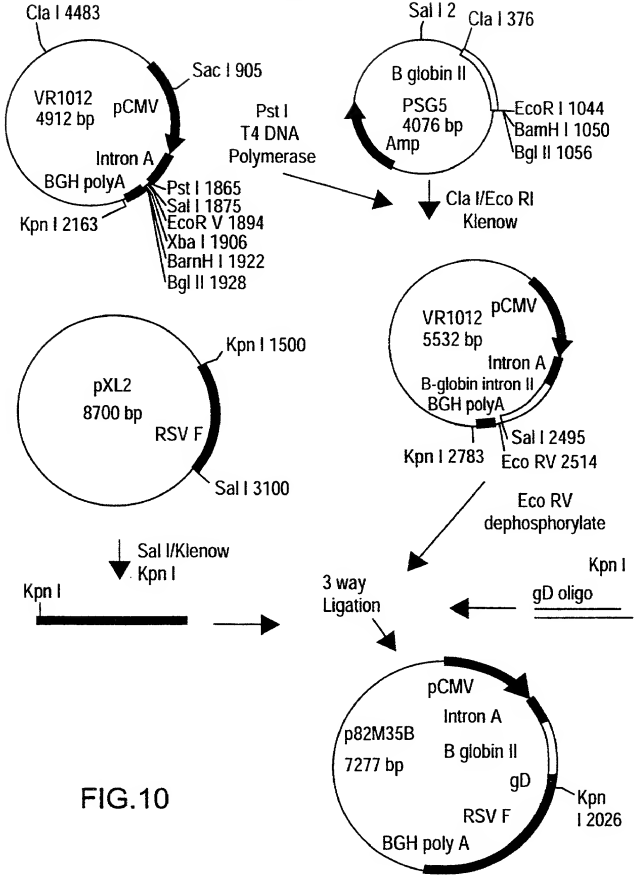
Cla I 4483
Sac I 905
VR1012
4912 bp
pCMV
Intron A
BGH polyA
Kpn I 2163
Pst I 1865
Sal I 1875
EcoR V 1894
Xba I 1906
BamH I 1922
Bgl II 1928
Sal I 2
Cla I 376
B globin II
PSG5
4076 bp
Amp
EcoR I 1044
BamH I 1050
Bgl II 1056
Pst I
T4 DNA
Polymerase
Cla I/Eco RI
Klenow
pXL2
8700 bp
RSV F
Kpn I 1500
Sal I 3100
VR1012
5532 bp
pCMV
Intron A
B-globin intron II
BGH polyA
Kpn I 2783
Sal I 2495
Eco RV 2514
Eco RV
dephosphorylate
Sal I/Klenow
Kpn I
Kpn I
3 way
Ligation
Kpn I
gD oligo
p82M35B
7277 bp
pCMV
Intron A
B globin II
gD
RSV F
BGH poly A
Kpn
I 2026

FIG.10

FIG.11A

Nucleotide Sequence of plasmid VR1012

```
                10                  20                  30                  40                  50                  60                  70
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA CAGCTTGTCT

                80                  90                 100                 110                 120                 130                 140
GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG

               150                 160                 170                 180                 190                 200                 210
CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA CCGCACAGAT

               220                 230                 240                 250                 260                 270                 280
GCGTAAGCGA AAAATACCGC ATCAGATTGG CTATTGGCCA TTGCATACGT TGTATCCATA TCATAATATG

               290                 300                 310                 320                 330                 340                 350
TACATTTATA TTGGCTCATG TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT

               360                 370                 380                 390                 400                 410                 420
AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT ACATAACTTA CGGTAAATGG

               430                 440                 450                 460                 470                 480                 490
CCCGCCTGGC TGACCGCCCA ACGACCCCCG CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG
```

FIG.11B

```
500                 510                 520                 530                 540                 550                 560
CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG GCAGTACATC

570                 580                 590                 600                 610                 620                 630
AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC

640                 650                 660                 670                 680                 690                 700
CCAGTACATG ACCTTATGGG ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG

710                 720                 730                 740                 750                 760                 770
GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT CCAAGTCTCC

780                 790                 800                 810                 820                 830                 840
ACCCCATTGA CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA

850                 860                 870                 880                 890                 900                 910
CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTCGTTTA

920                 930                 940                 950                 960                 970                 980
GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT TTGACCTCCA TAGAAGACAC CGGGACCGAT

990                 1000                1010                1020                1030                1040                1050
CCAGCCTCCG CGGCCGGAA CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC
```

09/914621

FIG.11C

```
1060       1070       1080       1090       1100       1110       1120
CTATAGACTC TATAGGCACA CCCCTTTGGC TCTTATGCAT GCTATACTGT TTTTGCCTTG GGGCCTATAC

1130       1140       1150       1160       1170       1180       1190
ACCCCCGCTT CCTTATGCTA TAGGTGATGG TATAGCTTAG CCTATAGGTG TGGGTTATTG ACCATTATTG

1200       1210       1220       1230       1240       1250       1260
ACCACTCCCC TATTGGTGAC GATACTTTCC ATTACTAATC CATAACATGG CTCTTTGCCA CAACTATCTC

1270       1280       1290       1300       1310       1320       1330
TATTGGCTAT ATGCCAATAC TCTGTCCTTC AGAGACTGAC ACGGACTCTG TATTTTTACA GGATGGGGTC

1340       1350       1360       1370       1380       1390       1400
CCATTTTATTA TTTACAAATT CACATATACA ACAACCCCGT CCCCCGTGCC CGCAGTTTTT ATTAAACATA

1410       1420       1430       1440       1450       1460       1470
GCGTGGGATC TCCACGCGAA TCTCGGGTAC GTGTTCCGGA CATGGGCTCT TCTCCCGTAG CGGCGAGCCT

1480       1490       1500       1510       1520       1530       1540
TCCACATCGG AGCCCTGGTC CCATGCCTCC AGCGGCTCAT GGTCGCTCGG CAGCTCCTTG CTCCTAACAG

1550       1560       1570       1580       1590       1600       1610
TGGAGGCCAG ACTTAGGCAC AGCACAATGC CCACCACCAC CAGTGTGCCG CACAGGCCGC TGGCGGTAGG
```

09/914621

FIG.11D

```
      1620       1630       1640       1650       1660       1670       1680
GTATGTGTCT GAAAATGAGC GTGGAGATTG GGCTCGCACG GCTGACGCAG ATGGAAGACT TAAGGCAGCG

      1690       1700       1710       1720       1730       1740       1750
GCAGAAGAAG ATGCAGGCAG CTGAGTTGTT GTATTCTGAT AAGAGTCAGA GGTAACTCCC GTTGCGGTGC

      1760       1770       1780       1790       1800       1810       1820
TGTTAACGGT GGAGGGCAGT GTAGTCTGAG CAGTACTCGT TGCTGCCGCG CGCGCCACCA GACATAATAG

      1830       1840       1850       1860       1870       1880       1890
CTGACAGACT AACAGACTGT TCCTTTCCAT GGGTCTTTTC TGCAGTCACC GTCGTCGACA CGTGTGATCA

      1900       1910       1920       1930       1940       1950       1960
GATATCGCGG CCGCTCTAGA CCAGGCGCCT GGATCCAGAT CTGCTGTGCC TTCTAGTTGC CAGCCATCTG

      1970       1980       1990       2000       2010       2020       2030
TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG TGCCACTCCC ACTGTCCTTT CCTAATAAAA

      2040       2050       2060       2070       2080       2090       2100
TGAGGAAATT GCATCGCATT GTCTGAGTAG GTGTCATTCT ATTCTGGGGG GTGGGGTGGG GCAGGACAGC

      2110       2120       2130       2140       2150       2160       2170
AAGGGGGAGG ATTGGGAAGA CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGGT ACCCAGGTGC
```

FIG.11E

```
2180      2190      2200      2210      2220      2230      2240
TGAACAATTG ACCCCGTTCC TCCTGGCCCA GAAAGAAGCA GGCACATCCC CTTCTCTGTG ACACACCCTG

2250      2260      2270      2280      2290      2300      2310
TCCACGCCCC TGGTTCTTAG TTCCAGCCCC ACTCATAGGA CACTCATAGC TCAGGAGGGC TCCGCCTTCA

2320      2330      2340      2350      2360      2370      2380
ATCCCACCCG CTAAAGTACT TGGAGCGGTC TCTCCCTCCC TCATCAGCCC ACCAAACCAA ACCTAGCCTC

2390      2400      2410      2420      2430      2440      2450
CAAGAGTGCG AAGAAATTAA AGCAGATAG GCTATTAAGT GCAGAGGGAG AGAAAATGCC TCCAACATGT

2460      2470      2480      2490      2500      2510      2520
GAGGAAGTAA TCAGAGAAAT CATAGAATTT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT

2530      2540      2550      2560      2570      2580      2590
TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC AGGGGATAAC

2600      2610      2620      2630      2640      2650      2660
GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT

2670      2680      2690      2700      2710      2720      2730
TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC
```

FIG.11F

```
2740      2750      2760      2770      2780      2790      2800
GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG

2810      2820      2830      2840      2850      2860      2870
CCGCTTACGG GATACCTGTC CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA

2880      2890      2900      2910      2920      2930      2940
GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA

2950      2960      2970      2980      2990      3000      3010
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA

3020      3030      3040      3050      3060      3070      3080
GCAGCCGACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC

3090      3100      3110      3120      3130      3140      3150
CTAACTACGG CTACACTAGA AGAACAGTAT TTGGTATCTG CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA

3160      3170      3180      3190      3200      3210      3220
AAGAGTTGGT AGCTCTTGAT CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

3230      3240      3250      3260      3270      3280      3290
CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT
```

FIG.11G

```
3300       3310       3320       3330       3340       3350       3360
GPAACGPAAA CTCACGTTAA GGGATTTTGG TCATGAGATT ATCPAAAAGG ATCTTCACCT AGATCCTTTT

3370       3380       3390       3400       3410       3420       3430
AAATTPAAAA TGAAGTTTTA AATCPATCTA AAGTATATAT GAGTPAAACTT GGTCTGPCAG TTACCPATGC

3440       3450       3460       3470       3480       3490       3500
TTPAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT AGTTGCCTGA CTCCCGGGGG

3510       3520       3530       3540       3550       3560       3570
GGGGGCGCTG AGGTCTGCCT CGTPAGPAAG GTGTTGCTGA CTCATACCAG GCCTGPATCG CCCCATCATC

3580       3590       3600       3610       3620       3630       3640
CAGCCAGPAA GTGPGGGPGC CACGGTTGAT GAGAGCTTTG TTGTAGGTGG ACCAGTTGGT GATTTTGPAC

3650       3660       3670       3680       3690       3700       3710
TTTTGCTTTG CCACGGPAGG GTCTGCGTTG TCGGGPAGAT GCGTGATCTG ATCCTTCPAC TCAGCPAAAG

3720       3730       3740       3750       3760       3770       3780
TTCGATTTAT TCAPCPAGC CGCCGTCCCG TCAAGTCAGC GTPAATGCTCT GCCAGTGTTA CPACCPATTA

3790       3800       3810       3820       3830       3840       3850
ACCPATTGTG ATTPGPAAAA CTCATCGAGC ATCPAATGPA ACTGCPATTT ATTCATPATCA GPATTATCPA
```

FIG.11H

```
3860       3870       3880       3890       3900       3910       3920
TACCATATTT TTCAAAAAGC CGTTTCTGTA ATGAAGCAGA AAACTCACCG AGCCAGTTCC ATAGCATGGC

3930       3940       3950       3960       3970       3980       3990
AAGAATCCTGG TATCGGTCTG CGATTCCGAC TCGTCCAACA TCAATACAAC CTATTAATTT CCCCTCGTCA

4000       4010       4020       4030       4040       4050       4060
AAAAATAGGT TATCAAGTGA GAAATCACCA TGAGTGACGA CTGAATCCGG TGAGAATGGC AAAAGCTTAT

4070       4080       4090       4100       4110       4120       4130
GCATTTCTTT CCAGACTTGT TCAACAGCCC AGCCATTACG CTCGTCATCA AAATCACTCG CATCAACCAA

4140       4150       4160       4170       4180       4190       4200
ACCGTTATTC ATTCGTGATT GCGCCTGAGC GAGACGAAAT ACGCGATCGC TGTTAAAAGG ACAATTACAA

4210       4220       4230       4240       4250       4260       4270
ACAGGAATCG AATGCAACCG GCGCAGGAAC ACTGCCAGCG CATCAACAAT ATTTTCACCT GAATCAGGAT

4280       4290       4300       4310       4320       4330       4340
ATTCTTCTAA TACCTGGAAT GCTGTTTTCC CGGGCATCGC AGTGGTGAGT AACCATGCAT CATCAGGAGT

4350       4360       4370       4380       4390       4400       4410
ACGGATAAAA TGCTTGATGG TCGGAAGAGG CATAAATTCC GTCAGCCAGT TTAGTCTGAC CATCTCATCT
```

FIG.11I

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      4420       4430       4440       4450       4460       4470       4480
GTAACATCAT TGGCAACGCT ACCTTTGCCA TGTTTCAGAA ACAACTCTGG CGCATCGGGC TTCCCATACA

      4490       4500       4510       4520       4530       4540       4550
ATCGATAGAT TGTCGCACCT GATTGCCCGA CATTATCGCG AGCCCATTTA TACCCATATA AATCAGCATC

      4560       4570       4580       4590       4600       4610       4620
CATGTTGGAA TTTAATCGCG GCCTCGAGCA AGACGTTTCC CGTTGAATAT GGCTCATAAC GTTCCTTGTA

      4630       4640       4650       4660       4670       4680       4690
TTACTGTTTA TGTAAGCAGA CAGTTTTATT GTTCATGATG ATATATTTTT ATCTTGTGCA ATGTAACATC

      4700       4710       4720       4730       4740       4750       4760
AGAGATTTTG AGACACAACG TGGCTTTCCC CCCCCCCCCA TTATTGAAGC ATTTATCAGG GTTATTGTCT

      4770       4780       4790       4800       4810       4820       4830
CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA

      4840       4850       4860       4870       4880       4890       4900
AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA

      4910
GGCCCTTTCG TC
```

FIG. 12

5'AAG CTT CAG GAA CGA CCA ACT ACC CCG ATC ATC AGT TAT CCT

TAA GGT CTC TTT TGT GTG GTG CGT TCC GGT ATG GGG GGG ACT GCC

Met Gly Gly Thr Ala

GCC AGG TTG GGG GCC GTG ATT TTG TTT GTC GTC ATA GTG GGC CTC

Ala Arg Leu Gly Ala Val Ile Leu Phe Val Val Ile Val Gly Leu

CAT GGG GTC CGC GGC AAA TAT GCC TTG GCG GAT GCC TCT CTC 3'

His Gly Val Arg Gly Lys Tyr Ala Leu Ala Asp Ala Ser Leu

Docket No.
1038-1191MIS:jb

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

the specification of which
(check one)

- ☐ is attached hereto.
- ☒ was filed on **March 3, 2000** as United States Application No. or PCT International Application Number **PCT/CA00/00227**
- and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

09/262,927	March 5, 1999	Pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/CA00/00227	March 3, 2000	
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Fifth inventor's signature — Date

Residence

Citizenship

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Full name of sixth inventor, if any

Sixth inventor's signature — Date

Residence

Citizenship

Post Office Address

Docket No.
1038-1191MIS:jb

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

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the specification of which
(check one)

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Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

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(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

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(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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